

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|--|-----------|---|
| (51) International Patent Classification ⁵ : A01N 43/16, A61K 31/35 | A1 | (11) International Publication Number: WO 93/18652 (43) International Publication Date: 30 September 1993 (30.09.93) |
| (21) International Application Number: PCT/US93/02378 (22) International Filing Date: 12 March 1993 (12.03.93) (30) Priority data: 854,167 20 March 1992 (20.03.92) US (71) Applicant: MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). (72) Inventors: HOLT, Tom, G. ; 779 Summit Avenue, Westfield, NJ 07090 (US). MONAGHAN, Richard, L. ; 48 Johnson Road, Somerset, NJ 08873 (US). (74) Agent: MUTHARD, David, A.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). | | (81) Designated States: AU, BB, BG, BR, CA, CZ, FI, HU, JP, KR, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG). Published <i>With international search report.</i> <i>With amended claims and statement.</i> |
| (54) Title: METHODS OF ACHIEVING ANTITUMOR ACTIVITY USING 16-MEMBERED-MACROLIDE-TYPE COMPOUNDS (57) Abstract Inhibition of renal cancer, melanoma, colon cancer, lung cancer and leukemia cells by administration of compounds from the bafilomycin and hygrolidin groups of compounds. | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria | FR | France | MR | Mauritania |
| AU | Australia | GA | Gabon | MW | Malawi |
| BB | Barbados | GB | United Kingdom | NL | Netherlands |
| BE | Belgium | GN | Guinea | NO | Norway |
| BF | Burkina Faso | GR | Greece | NZ | New Zealand |
| BG | Bulgaria | HU | Hungary | PL | Poland |
| BJ | Benin | IE | Ireland | PT | Portugal |
| BR | Brazil | IT | Italy | RO | Romania |
| CA | Canada | JP | Japan | RU | Russian Federation |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SD | Sudan |
| CG | Congo | KR | Republic of Korea | SE | Sweden |
| CH | Switzerland | KZ | Kazakhstan | SK | Slovak Republic |
| CI | Côte d'Ivoire | LJ | Liechtenstein | SN | Senegal |
| CM | Cameroon | LK | Sri Lanka | SU | Soviet Union |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | MC | Monaco | TG | Togo |
| DE | Germany | MG | Madagascar | UA | Ukraine |
| DK | Denmark | ML | Mali | US | United States of America |
| ES | Spain | MN | Mongolia | VN | Viet Nam |
| FI | Finland | | | | |

- 1 -

TITLE OF THE INVENTION
METHODS OF ACHIEVING ANTITUMOR ACTIVITY
USING 16-MEMBERED-MACROLIDE-TYPE COMPOUNDS

5 **FIELD OF THE INVENTION**

The invention pertains to the inhibition of certain types of cancerous cells. More specifically, renal cancer, melanoma, lung cancer, colon cancer and leukemia cells are inhibited by administration of compounds from the groups of bafilomycin and hygrolidin and
10 analogs of each of them.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a type of bar chart known as a mean graph, showing the Total Growth Inhibition caused by compound number 4
15 from the table below, upon a panel of some 60 tumor cell lines.

Figure 2 is the same type of chart as in Figure 1, showing the Total Growth Inhibition caused by compound number 16 from the table below.

20

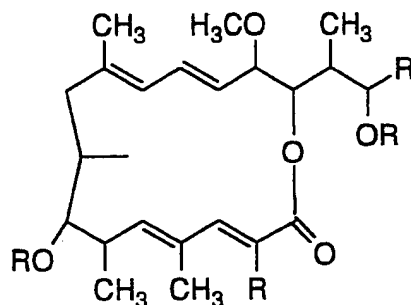
25

30

- 2 -

BACKGROUND OF THE INVENTION

The aforementioned bafilomycins and hygrolidins (which collectively are known as hygrolides) were previously known and shown to have activity as antiparasitics, anthelmintics, antibiotics, insecticides, antifungals, agents for the therapy of bone diseases such as osteoporosis and as Na^+ , K^+ ATP-ase inhibitors leading to increased cardiac function. These compounds share the structural feature of having a 16-membered macrocyclic lactone ring:



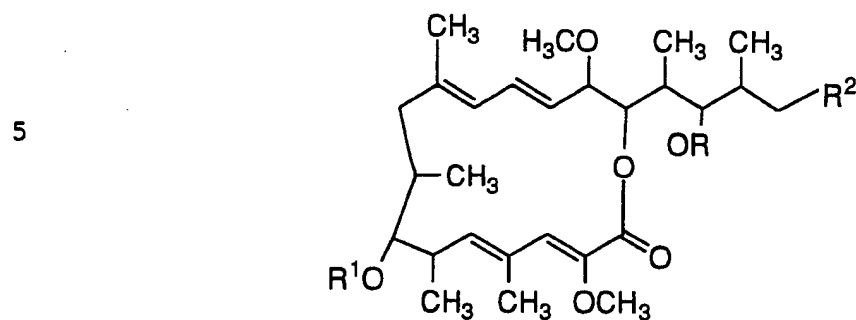
When run through whole tumor-cell assays, compounds from this group have been found to exhibit antitumor activity.

SUMMARY OF THE INVENTION

The invention here is a method of obtaining antitumor activity, in particular against cancers taken from the group consisting of melanoma, renal cancer, lung cancer, colon cancer and leukemia, by administration to mammalian tumor cells of a pharmacologically effective amount of a compound selected from the group consisting of

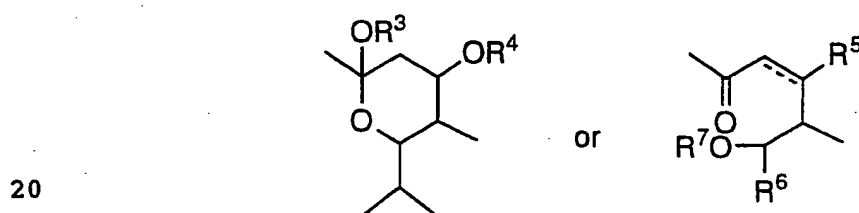
- 3 -

(a) a bafilomycin of the formula



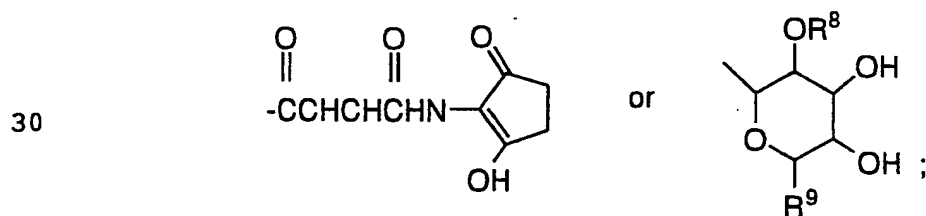
where R is hydrogen or acetyl, R¹ is hydrogen or the acyl radical of a carboxylic acid having one to six carbon atoms;

15 R² is



R³ is hydrogen or methyl;

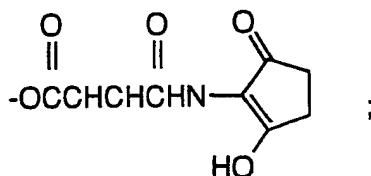
25 R⁴ is hydrogen, methyl, the acyl radical of a carboxylic acid having one to six carbon atoms,



- 4 -

R⁵ is hydrogen or

5



R⁶ is methyl or isopropyl;

10

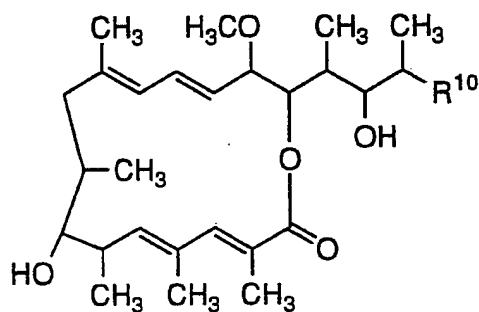
R⁷ is hydrogen or acetate;

R⁸ is hydrogen or methyl;

R⁹ is hydroxy or methyl; and

(b) a hygrolidin of the formula

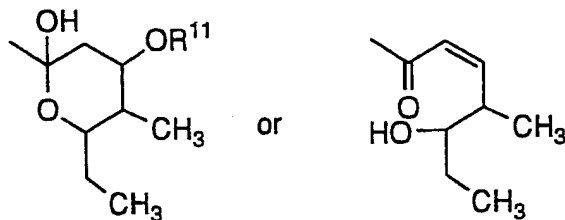
15



20

where R¹⁰ is

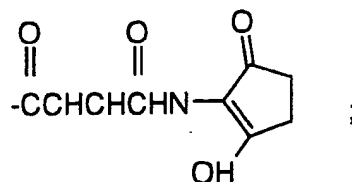
25



30

and R¹¹ is hydrogen, $-\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{NH}_2$, $-\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OH}$ or

- 5 -



5

The term "pharmacologically effective amount" shall mean that amount of a drug or pharmaceutical agent that will elicit the biological or medical response of a tissue, system, animal or human that is being sought by a researcher or clinician.

The compounds of the present invention can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixers, tinctures, suspensions, syrups and emulsions. Likewise, they may also be administered in intravenous form (both bolus and infusion), intraperitoneal, subcutaneous or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. An effective but non-lethal amount of the compound desired can be employed as an antitumor agent.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound or salt thereof employed. An ordinarily skilled physician or veterinarian can readily determine and prescribe the effective amount of the drug required.

Oral dosages of the present invention, when used for the indicated effects, will range between about 1-25 mg/day orally. Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, preferred compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal

- 6 -

vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

In the methods of the present invention, the compounds herein described in detail can form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and the like.

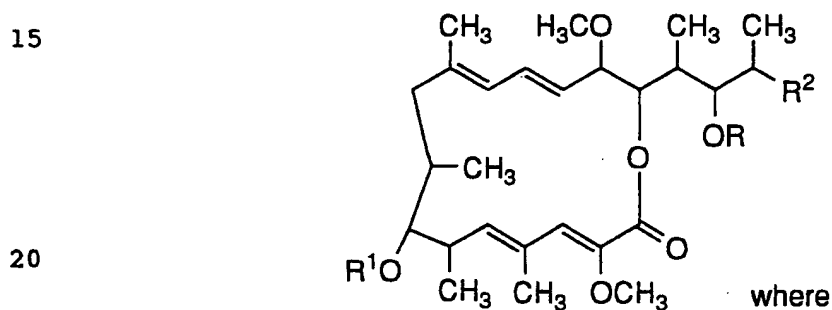
The compounds of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

Compounds of the present invention may also be delivered by the use of monoclonal antibodies as individual carriers to which the compound molecules are coupled. The compounds of the present

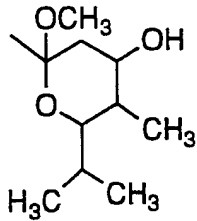
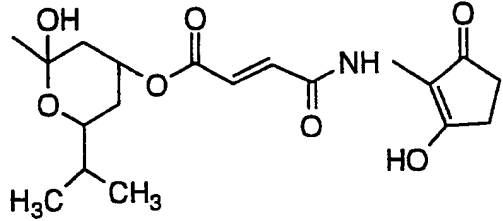
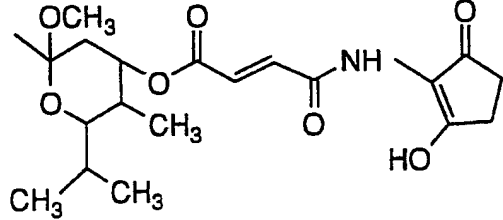
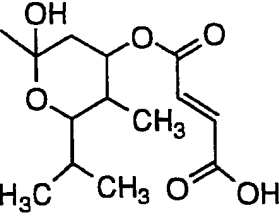
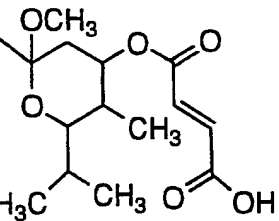
- 7 -

invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxypropylmethacrylamidephenol, polyhydroxyethylaspartamidephenol, or polyethyleneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers or hydrogels.

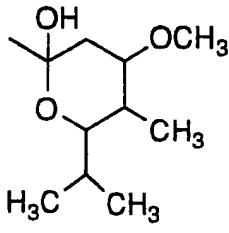
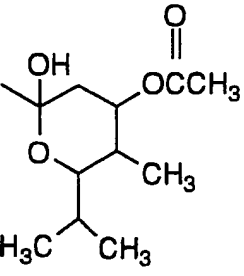
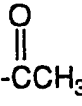
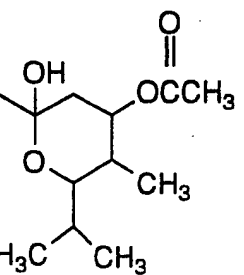
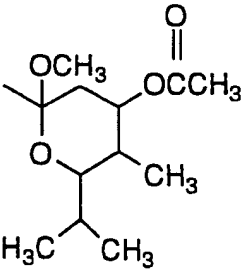
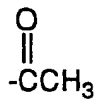
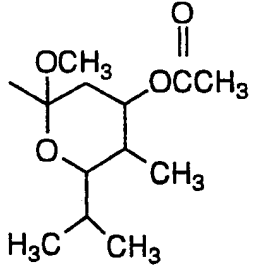
Preferred bafilomycins used in the method of the invention include the following:



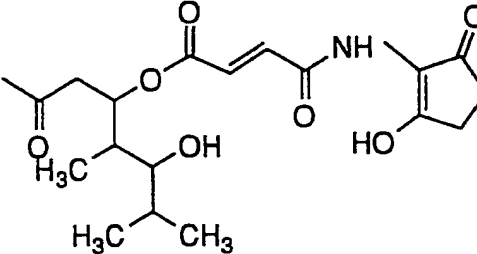
- 8 -

| | <u>Compound</u> | <u>R¹</u> | <u>R²</u> |
|----|-----------------|----------------------|--|
| 5 | (1) | H |  |
| 10 | (2) | H |  |
| 15 | (3) | H |  |
| 20 | (4) | H |  |
| 25 | (5) | H |  |
| 30 | | | |

- 9 -

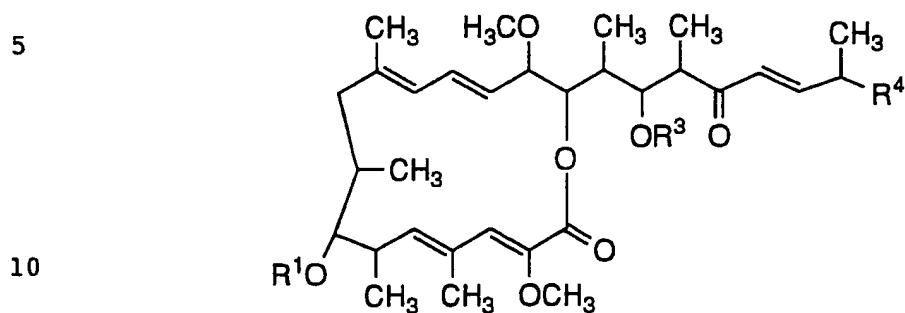
| | <u>Compound</u> | <u>R¹</u> | <u>R²</u> |
|----|-----------------|---|--|
| 5 | (6) | H |  |
| 10 | (7) | H |  |
| 15 | (8) |  |  |
| 20 | (9) | H |  |
| 25 | (10) |  |  |
| 30 | | | |

- 10 -

| | <u>Compound</u> | <u>R¹</u> | <u>R²</u> |
|----|-----------------|----------------------|--|
| 5 | (11) | H |  |
| 10 | | | |
| 15 | | | |
| 20 | | | |
| 25 | | | |
| 30 | | | |

- 11 -

Another group of preferred compounds are set forth as follows:

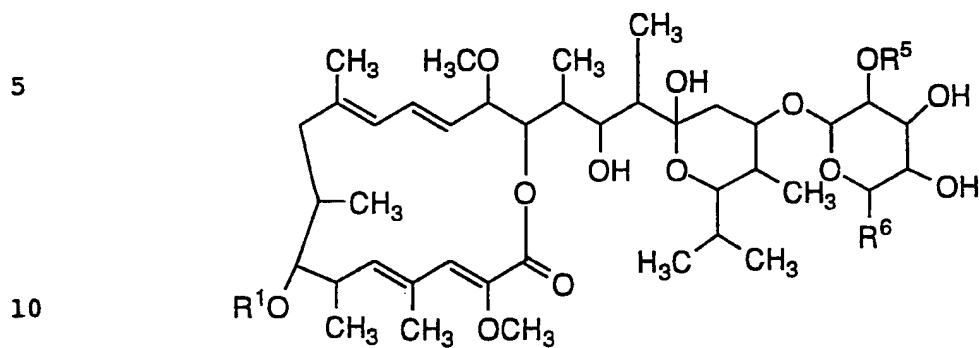


| Compound | R^1 | R^3 | R^4 |
|------------|-------|-------|-------|
| 15 (12) | H | H | |
| 20 (13) | H | H | |
| 25 (14) | | | |

30

- 12 -

Some compounds in the bafilomycin class are sometimes referred to as leucanicidins. Examples include the following:



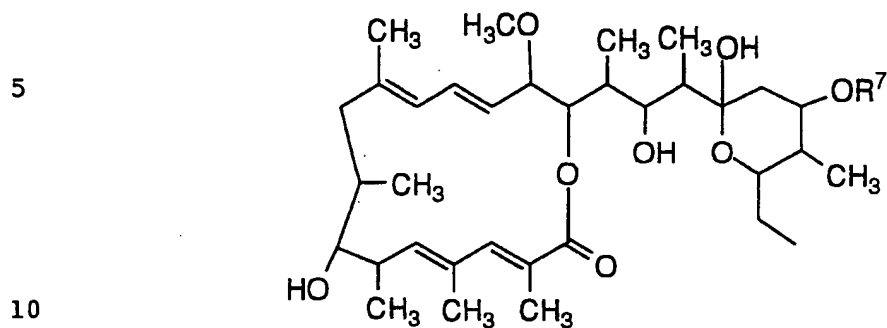
| Compound | <u>R¹</u> | <u>R⁵</u> | <u>R⁶</u> |
|----------|---|----------------------|----------------------|
| (15) | H | -CH ₃ | CH ₃ |
| (16) | $\text{C}(=\text{O})\text{CH}(\text{CH}_3)_2$ | CH ₃ | CH ₃ |
| (17) | H | H | -OH |
| (18) | H | H | -CH ₃ |

25

30

- 13 -

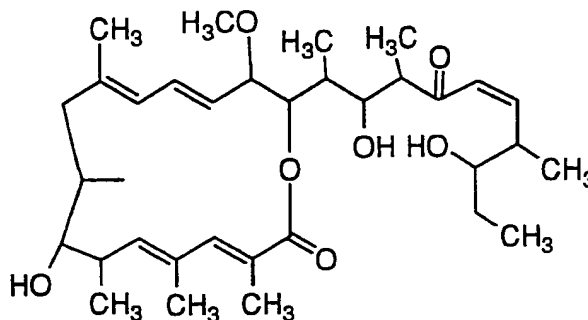
Preferred hygrolidins used in the method of the invention include the following



| <u>Compound</u> | <u>R⁷</u> |
|-----------------|----------------------|
| (19) | |
| (20) | |
| (21) | |
| (22) | H |

- 14 -

An additional preferred compound is oxyhygrolidin:



The most preferred compounds of the invention are any or all of those specifically set forth in these examples. These compounds are not, however, to be construed as forming the only genus that is considered as the invention, and any combination of the compounds of their moieties may itself form a genus.

Methods of making bafilomycins are taught in U.S. Patent Nos. 4,385,065 and 4,558,139 and in Kretschmer et al. Agric. Biol. Chem., 49.8 (1985) 2509-2511. Methods of making leucanicidins are taught in Meyer et al, Helv. Chim. Acta. 68 (1985) 83-94. Methods of making hygrolidins are taught in U.S. Patent No. 4,390,546 and in Kretschmer, et al, ibid. The entire disclosure of these patents and journal articles is incorporated herein by reference, and variations of the methods disclosed therein are readily made by those of ordinary skill in the art.

DETAILED DESCRIPTION OF THE INVENTION

An in vitro human tumor drug screen is used to evaluate compounds used in the method of the present invention. The screen employed has been described and the experimental details are taught in the text and bibliography of Boyd, et al., Status of the NCI Preclinical Antitumor Drug Discovery Screen; Principles and Practices of Oncology, Vol. 3, No. 10, Oct. 1989, Lippincott. The screen is a disease-oriented primary screen that employs multiple disease-specific (i.e. tumor-type specific) models. The models are based on the

- 15 -

availability of a wide variety of human tumor cell lines, representing many different forms of human cancer. These cell lines comprise a diverse panel of human tumor cell lines arrayed in disease-specific subpanels.

5 The panel contains a total of 60 human tumor cell lines derived from seven cancer types (lung, colon, melanoma, renal, ovarian, brain and leukemia) that have been adapted to a single growth medium and that have reproducible profiles for growth and drug
10 sensitivity.

EXAMPLE 1

 Individual cell lines were initially photographed, expanded
15 (two passages maximum) and cryo-preserved (master stocks) with growth medium and split-ratios recommended by their respective sources. Only cell lines documented to be free of adventitious bacteria and pathogenic viruses (NCI-FCRF³ Diagnostic Microbiology Lab and Animal Health Diagnostic Lab) were accepted for subsequent
20 characterization. Following recovery of master stocks, cell lines were adapted to a single, standard culture medium: RPMI 1640 (Quality Biologicals, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Sterile Systems Hyclone, Logan, UT) and 2 mM L-glutamine (NCI-FCRF Central Medium Laboratory) without antibiotics and cultured under conventional culture conditions,
25 that is, 37°C, 5% CO₂, 95% air, 100% relative humidity. Cell lines were then expanded (five passages maximum) and cryopreserved for generation of seed stocks. Established adherent cell monolayers approaching 80% confluency were harvested with trypsin/EDTA (NCI-FCRF Central Medium Laboratory) whereas some early passage
30 adherent cell lines were harvested with solution A and 2X-crystalized trypsin III (Sigma Chemical Co.). Leukemia cell lines were subcultured by trituration and dilution. Small cell lung carcinoma cell lines (which generally form large aggregates in suspension under conventional culture conditions) were cultured and assayed in suspension as well as

- 16 -

adherent monolayers utilizing poly-L-lysine pretreatment of culture vessels. Following recovery of seed stock, cell lines were subjected to isoenzyme analysis as well as preliminary growth and drug sensitivity assays using one or more in vitro growth inhibition assays (described
5 below). Cell lines meeting basic quality assurance criteria (mycoplasma-negative, MAP-negative, human isoenzymes only) and exhibiting suitable growth profiles were expanded (five serial passages from seed stock, maximum) and cryopreserved as a large number of aliquots designated working seed stock. Cell cryopreservation was
10 achieved using a CryoMed controller (Model 801) and a CryoMed freezing chamber (No. 2700) with a step rate of $-1^{\circ}\text{C}/\text{min}$ followed by storage in vapor-phase liquid N_2 (NCI-FCRF Central Repository). Cell line seed stocks were tested also for in vivo tumorigenicity (s.c. and i.p. inoculations) in accordance with established protocols. Cell lines
15 recovered from working seed stocks were subjected to repeat mycoplasma tests and to more extensive in vitro growth characterization. Cell lines subsequently were evaluated with respect to stability in drug sensitivity profiles over the course of 20 weekly passages. In addition, each cell line was expanded (eight passages,
20 maximum from seed stock thaw) and cryopreserved as a large number of aliquots ("roller bottle" stock) for in vivo characterization and assay development.

Tetrazolium/formazan reagents were purchased from Sigma Chemical Co. (St. Louis, MO): MTT (m2128), MTT formazan (M2003), INT (18377), INT formazan (I7375), and NBT (N6876).
25 DMSO was purchased from Sigma Chemical Co. (D5879), J. T. Baker Chemical Co. (9194-3, Phillipsburg, NJ), and American Burdick and Jackson Laboratories (Spectrophotometric Grade Product 081, Muskegan, MI). These chemicals were stored in unopened bottles at
30 room temperature in the dark or in 50-ml sterile plastic tubes at -20°C in the dark. Anhydrous isopropanol (505-7) and propylene glycol (P-1009) were purchased from Sigma Chemical Co. Reagent grade hydrochloric acid (A-744) and hexane (H-302-1) were purchased from Fisher Scientific Co. Dimethylformamide (27,054-7) was purchased

- 17 -

from Aldrich Chemical Co. (Milwaukee, WI) Propanol (spectrophotometric grade 9068-1) was purchased from American Burdick and Jackson Laboratories. Crystalline stock materials were stored at -20°C. Solvent-reconstituted chemotherapeutic agents were
5 prepared at high concentration, partitioned into multiple aliquots, and stored at -70°C. Just prior to culture application the contents of frozen vials were thawed and mixed. Measured aliquots (20-200 µl) were transferred by micropipet (Gilson Pipetman, Models P200 and P1000) equipped with polypropylene tips to standard culture medium within
10 polypropylene tubes (Sarstedt 62.554/002 or Falcon 2098) and serially diluted in culture medium containing an appropriate concentration of vehicle. In principle, the viable cell number/well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically. In brief, cells
15 were harvested from exponential-phase maintenance cultures (T-75 cm² flasks; Falcon Plastics 3023), counted by trypan blue exclusion, and dispensed within replicate 96-well culture plates (Falcon Plastics 3075) in 100-µl volumes using a repeating pipet (Eppendorf repeater 4780) or multichannel pipet (Flow Labs, Titertek). Following a 24-h incubation
20 at 37°C, 5% CO₂, 100% relative humidity (Heraeus B5060EKO2 incubators or NAPCO 5300 incubators), 100 µl of culture medium, culture medium containing drug or culture medium containing drug vehicle was dispensed within appropriate wells (vehicle control group, N = 6; each drug treatment group, N = 3). Peripheral wells of each
25 plate (lacking cells) were utilized for drug blank (N = 2) and medium/tetrazolium reagent blank (N = 6) "background" determinations. Culture plates were then incubated for 1 to 11 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ml PBS (Quality Biologicals, Inc.) was
30 sterile filtered with 0.45-µm filter units (Nalgene type SCN) and stored at 4°C for a maximum of 1 month. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in prewarmed standard culture medium. Alternatively, other tetrazolium reagents (namely, INT or NBT) were

- 18 -

prepared and utilized in a similar fashion for selected experiments. Under standard MTA conditions 50 ml of MTT working solution was added to each culture well (resulting in 50 μ g MTT/250 μ l to total medium volume) and cultures were incubated at 37°C for 4 to 24 h depending upon individual cell line requirements. Following incubation cell monolayers and formazan were inspected microscopically: Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All but 10-20 μ l of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-gauge needle and replaced with 150 μ l of DMSO (Burdick & Jackson) using a multichannel pipet. Following thorough formazan solubilization (trituration by pipet or vibration on a plate shaker), the absorbance of each well was measured using a microculture plate reader (Dynatech MR600; Alexandria, VA) at 540 nm (single wavelength, calibration factor = 1.00) interfaced with an Apple IIe computer. Subsequently, data were stored and analyzed through use of Apple Soft, Apple Turnover, and Lotus Symphony software. Cell line growth and growth inhibition were expressed in terms of mean (± 1 SD) absorbance units and/or percentage of control absorbance (± 1 SD%) following subtraction of mean "background" absorbance. Linearity and reproducibility of instrument measurements were verified by the use of formazan reagents in appropriate solvent systems.

Absorption spectra of formazan reagents as well as cell-generated formazans were measured with a UV/visible scanning spectrophotometer (Perkin-Elmer Lambda V; Perkin-Elmer Corp., Norwalk, CT). Samples were placed in 1-cm pathlength disposable polystyrene cuvetts (Fisher Scientific Co. 14-385-942) except those solubilized in dimethylformamide solvent which were evaluated in 1-cm pathlength glass spectrophotometer cells (Coleman S7300-4). Freshly prepared material was analyzed in dual beam mode with 2-nm slit width, at 120 nm/min. 0.02 A threshold, and 0.5 s response. Instrument wavelength calibration was verified by examination of deuterium emission spectra to be 653.1 ± 0.3 nm.

- 19 -

Following 1-11 days' incubation, supernatant culture medium was removed and 200 µl of methylene blue (Sigma MB-1) solution [5 g/liter in ethanol:water (50%, v/v)] was added without delay. Following incubation at room temperature for 45 min, unbound stain
5 was removed by plate inversion on absorbant paper and subsequent emersion/dilution in four, 1-liter washes with distilled deionized water. Bound protein stain was solubilized by the addition of 100 µl SDS (Sigma L4509) solution (1%, v/v in water) to each well. Absorbances of wells were measured at 630 nm (single wavelengths, calibration
10 factor = 1.00) using equipment and computerized analysis procedures described above for the MTA.

EXAMPLE 2

15 A more preferred method uses the anionic dye sulforhodamine B (SRB). SRB is a bright pink anionic dye that, in dilute acetic acid, binds electrostatically to the basic amino acids of fixed monolayers of cells. SRB can detect about 1000 cells at 560 nM and 200-300 cells fluorescently. Supermaximal concentration and staining
20 time were determined by dose-response and kinetic analyses. A more preferred medium comprises beta-glycerophosphate as the buffering agent, and the medium has a stable physiological pH of 7.4 in atmospheric CO₂. The medium includes biotin, L-asparagine, pyruvate and oxaloacetate for metabolic stimulation of intracellular CO₂
25 production.

EXAMPLE 3

Measures of relative cell line sensitivities are done by
30 comparing relative drug concentrations required to produce the same level of response in each cell line visually. This is presented by a mean graph. Two mean graphs appear at Figures 1 and 2. The mean graph, derived from Total Growth Inhibition (TGI) values, is centered at the arithmetic mean of the logarithm of the TGI values for all cell live

- 20 -

responses measured for a given compound. The mean graph is construed by projecting bars, one for each cell line (listed by identified on the left of the graph) to either the right or the left of the mean, depending on whether cell sensitivity to the test drug is greater or less than average. The length of a bar is proportional to the difference between the logarithm of the cell line TGI and the mean. For example, for a given cell line, a bar projecting three log units to the right of the mean would reflect an individual cellular sensitivity 1,000 times that of the average of all the cellular responses to the given compound represented on the graph.

Figures 1 and 2 demonstrate that compounds 4 and 19, respectively have especially good antitumor activity against colon cancer cells, renal cancer cells, melanoma cells, lung cancer cells (non-small cells) and leukemia cells. More limited activity is also shown against the other cell lines.

The extrapolation of positive results in the above-described assays to utility as antitumor agents is supported by Boyd, M.R., Status of the NCI Preclinical Antitumor Drug Discovery Screen; Principles and Practices of Oncology, Vol. 3, No. 10, October 1989, Lippincott, the entire disclosure of which is incorporated herein by reference.

25

30

- 21 -

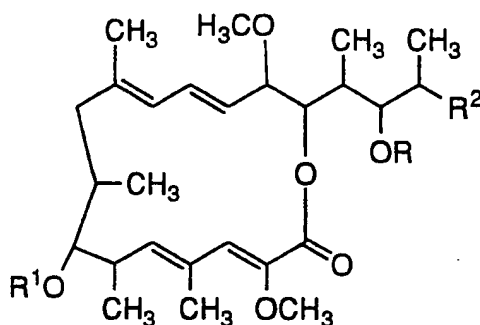
WHAT IS CLAIMED IS:

1. A method of treating tumors of renal cells, colon cells, lung cells or melanoma in a mammal in need thereof, comprising
 5 the step of administering to said mammal a pharmacologically effective amount of a compound selected from the group consisting of

(a) a bafilomycin of the formula

10

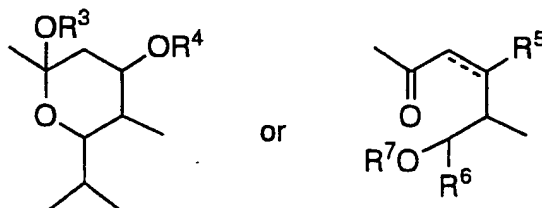
15



- 20 where R is hydrogen or acetyl, R¹ is hydrogen or the acyl radical of a carboxylic acid having one to six carbon atoms;

R² is

25

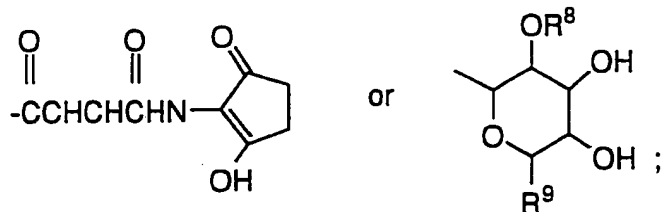


30

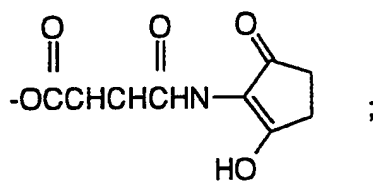
R³ is hydrogen or methyl;

R⁴ is hydrogen, methyl, the acyl radical of a carboxylic acid having one to six carbon atoms;

- 22 -

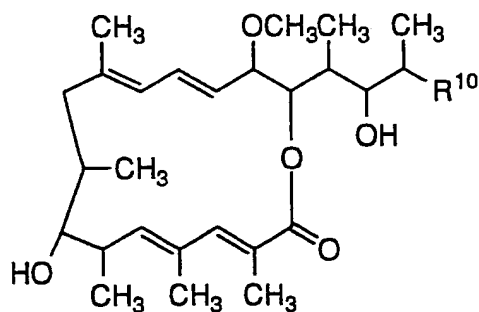


10 R^5 is hydrogen or



15 R^6 is methyl or isopropyl;
 R^7 is hydrogen or acetate;
 R^8 is hydrogen or methyl;
 R^9 is hydroxy or methyl; or

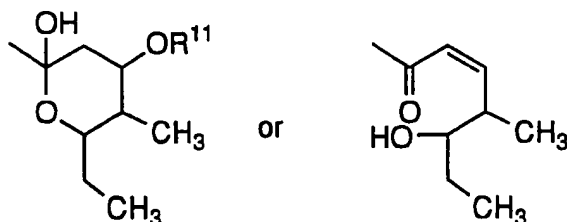
20 (b) a hygrolicidin of the formula



30 where R^{10} is

- 23 -

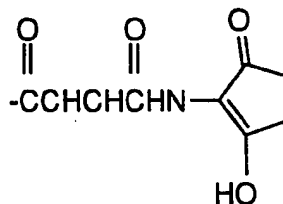
5



10

and R¹¹ is hydrogen, $\text{-C(=O)CHCH(=O)NH}_2$, -C(=O)CHCH(=O)OH or

15



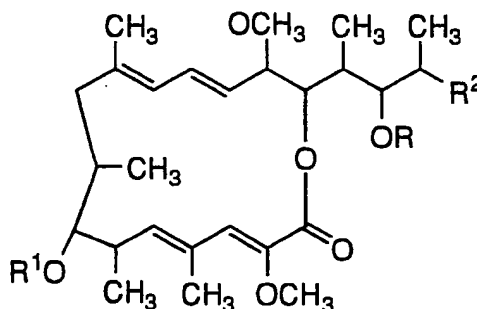
20

2. A method of treating tumors of renal cells in a mammal in need thereof, comprising the step of administering to said mammal a pharmacologically effective amount of a compound selected from the group consisting of

25

(a) a bafilomycin of the formula

30

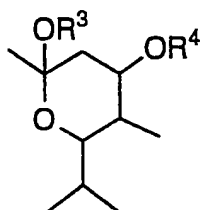


where R is hydrogen or acetyl, R¹ is hydrogen or the acyl radical of a carboxylic acid having one to six carbon atoms;

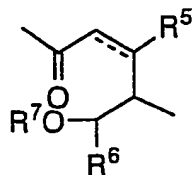
- 24 -

R² is

5



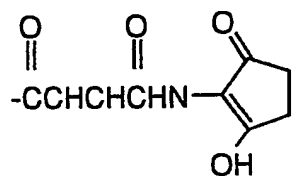
or



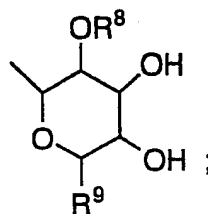
10

R³ is hydrogen or methyl;R⁴ is hydrogen, methyl, the acyl radical of a carboxylic acid having one to six carbon atoms;

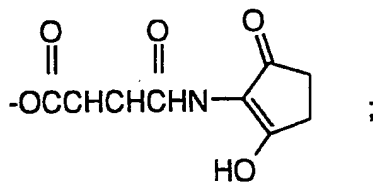
15



or

20 R⁵ is hydrogen or

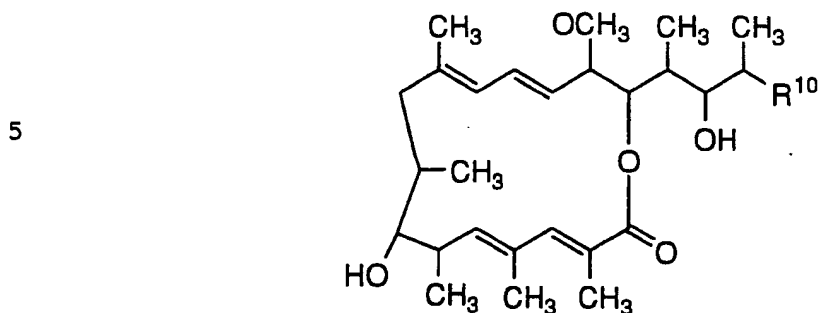
25



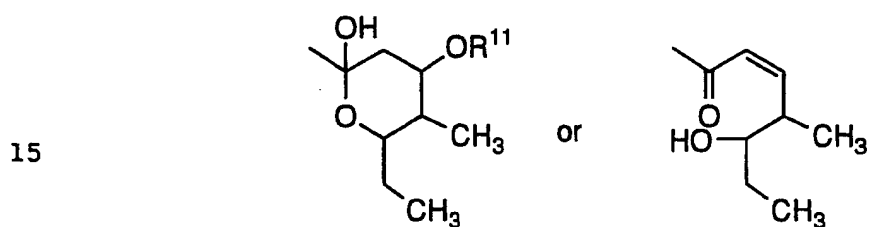
R⁶ is methyl or isopropyl;
 R⁷ is hydrogen or acetate;
 30 R⁸ is hydrogen or methyl;
 R⁹ is hydroxy or methyl; or

- 25 -

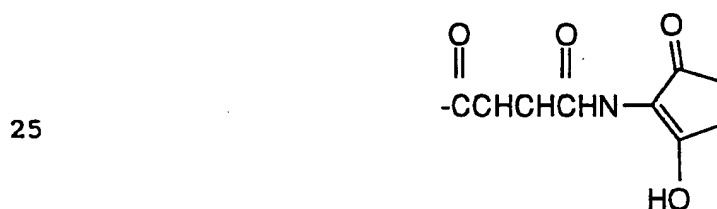
(b) a hygrolidin of the formula



10 where R¹⁰ is



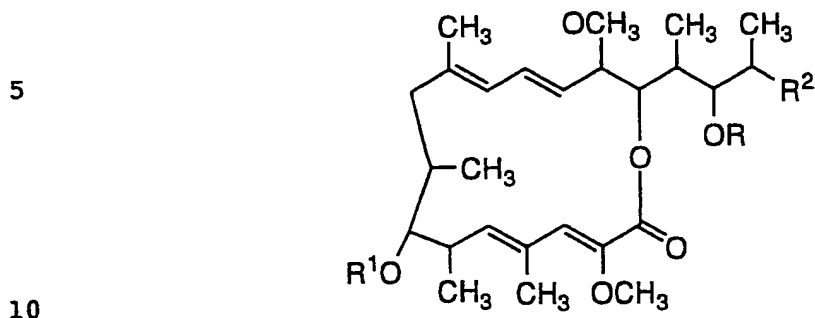
20 and R¹¹ is hydrogen, $-\overset{\text{O}}{\parallel}\text{CCHCHCNH}_2$, $-\overset{\text{O}}{\parallel}\text{CCHCHCOH}$ or



3. A method of treating tumors of colon cells, in a
 30 mammal in need thereof, comprising the step of administering to said
 mammal a pharmacologically effective amount of a compound selected
 from the group consisting of

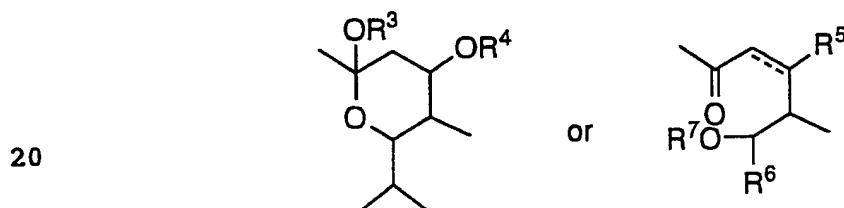
- 26 -

(a) a bafilomycin of the formula



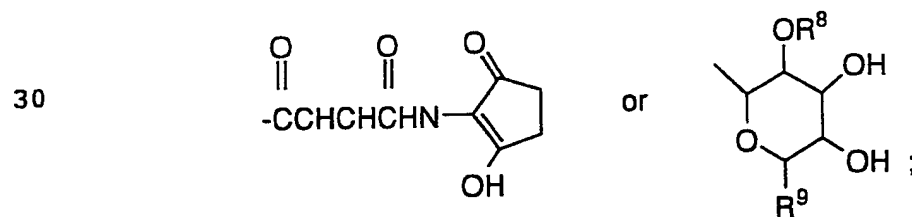
where R is hydrogen or acetyl, R¹ is hydrogen or the acyl radical of a carboxylic acid having one to six carbon atoms;

15 R² is



R³ is hydrogen or methyl;

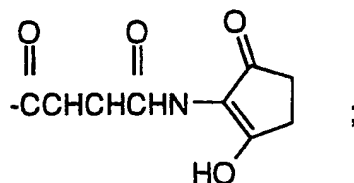
25 R⁴ is hydrogen, methyl, the acyl radical of a carboxylic acid having one to six carbon atoms;



- 27 -

R⁵ is hydrogen or

5



R⁶ is methyl or isopropyl;

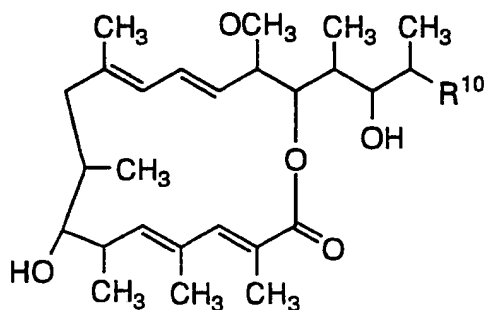
10 R⁷ is hydrogen or acetate;

R⁸ is hydrogen or methyl;

R⁹ is hydroxy or methyl; or

(b) a hygrolidin of the formula

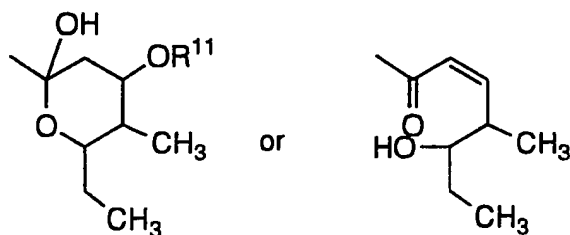
15



20

where R¹⁰ is

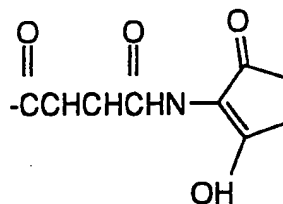
25



30

and R¹¹ is hydrogen, $\text{-C(=O)CH}_2\text{C(=O)NH}_2$, $\text{-C(=O)CH}_2\text{C(=O)OH}$ or

- 28 -



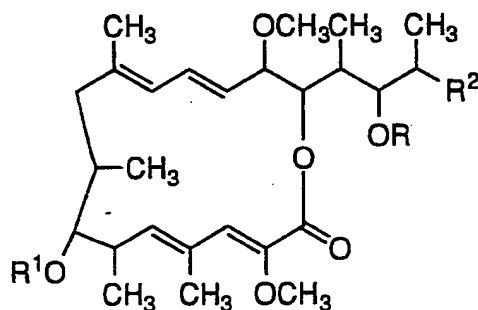
5

4. A method of treating tumors of lung cells in a mammal in need thereof, comprising the step of administering to said mammal a pharmacologically effective amount of a compound selected from the group consisting of

10

(a) a bafilomycin of the formula

15



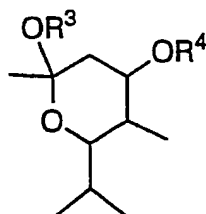
20

where R is hydrogen or acetyl, R¹ is hydrogen or the acyl radical of a carboxylic acid having one to six carbon atoms;

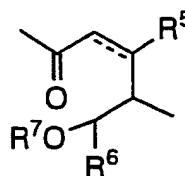
25

R² is

30



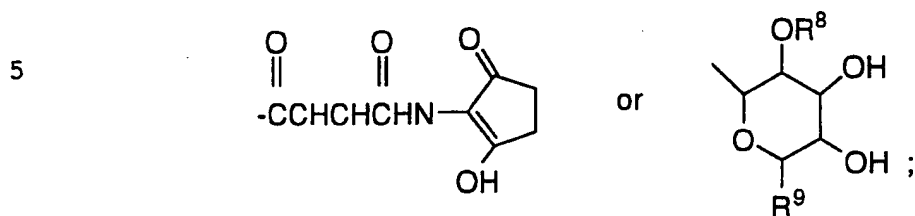
or



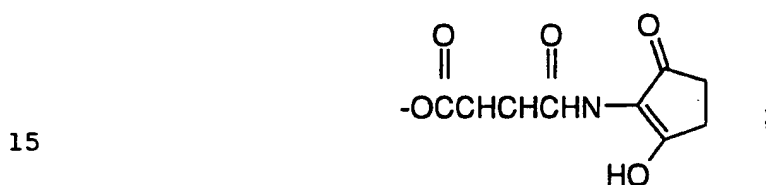
R³ is hydrogen or methyl;

- 29 -

R⁴ is hydrogen, methyl, the acyl radical of a carboxylic acid having one to six carbon atoms;



10 R⁵ is hydrogen or



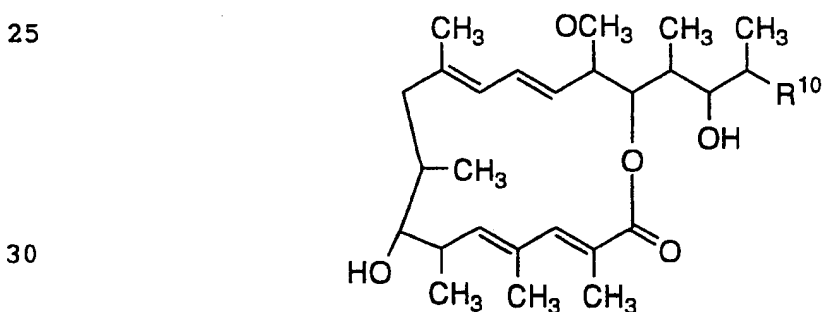
R⁶ is methyl or isopropyl;

R⁷ is hydrogen or acetate;

20 R⁸ is hydrogen or methyl;

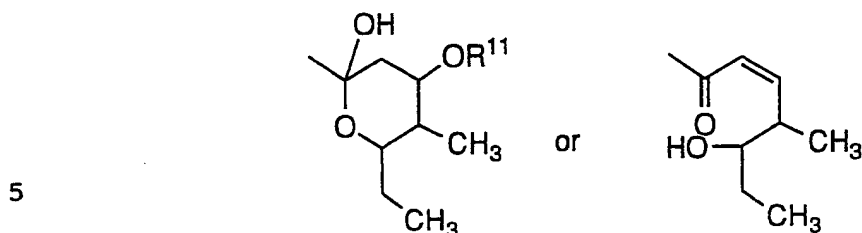
R⁹ is hydroxy or methyl; or

(b) a hygrolidin of the formula



where R¹⁰ is

- 30 -

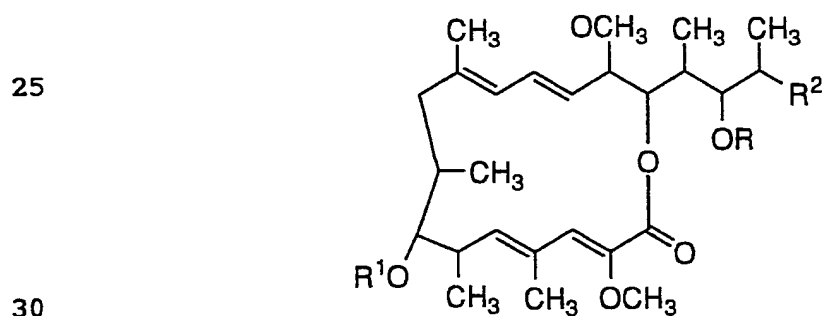


10 where R¹¹ is hydrogen, $\text{-}\overset{\text{O}}{\parallel}\text{CCHCH}\overset{\text{O}}{\parallel}\text{C}\text{NH}_2$, $\text{-}\overset{\text{O}}{\parallel}\text{CCHCH}\overset{\text{O}}{\parallel}\text{C}\text{OH}$



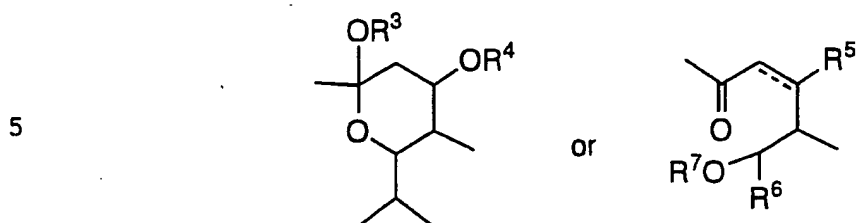
20 5. A method of treating tumors of melanoma in a mammal in need thereof, comprising the step of administering to said mammal a pharmacologically effective amount of a compound selected from the group consisting of

(a) a bafilomycin of the formula

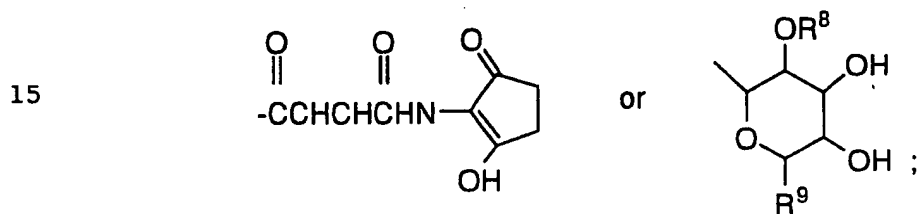


where R is hydrogen or acetyl, R¹ is hydrogen or the acyl radical of a carboxylic acid having one to six carbon atoms;

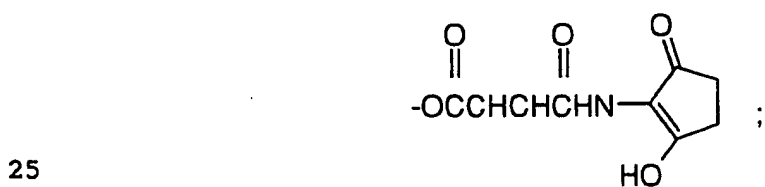
- 31 -

R² is

- 10 R³ is hydrogen or methyl;
 R⁴ is hydrogen, methyl, the acyl radical of a carboxylic acid having one to six carbon atoms;



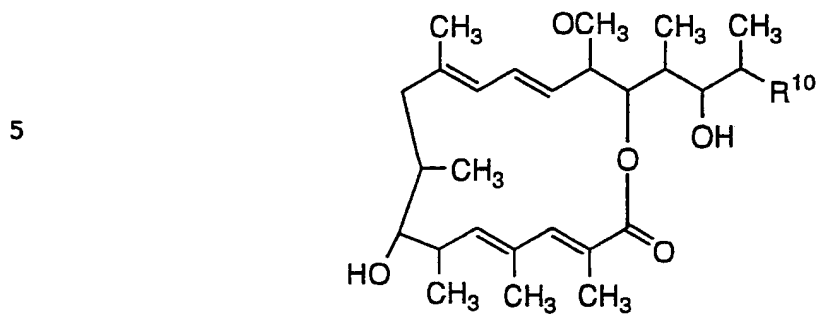
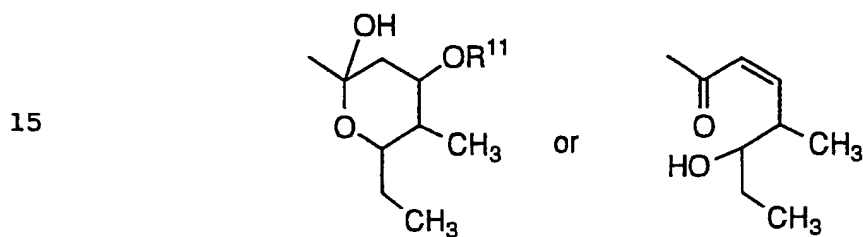
- 20 R⁵ is hydrogen or



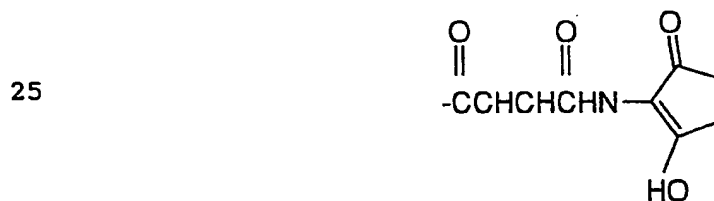
- 30 R⁶ is methyl or isopropyl;
 R⁷ is hydrogen or acetate;
 R⁸ is hydrogen or methyl;
 R⁹ is hydroxy or methyl; or

- 32 -

(b) a hygrolidin of the formula

where R¹⁰ is

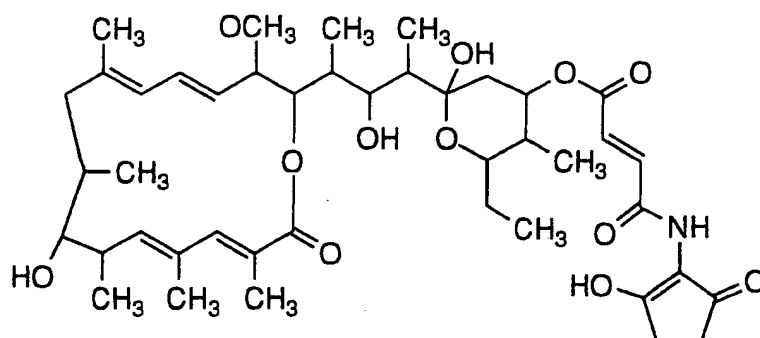
where R¹¹ is hydrogen, $-\overset{\text{O}}{\parallel}\text{CCHCH}\overset{\text{O}}{\parallel}\text{C}\text{NH}_2$, $-\overset{\text{O}}{\parallel}\text{CCHCH}\overset{\text{O}}{\parallel}\text{C}\text{OH}$



6. The method as claimed in Claim 1, wherein said compound administered to said mammal is

- 33 -

5

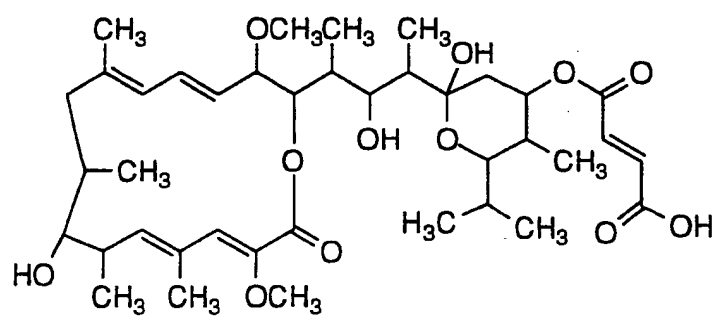


10

7. The method as claimed in Claim 1, wherein said compound administered to said mammal is

15

20



25

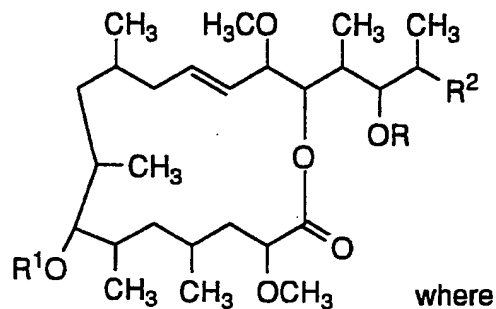
30

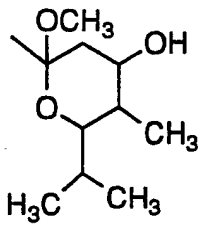
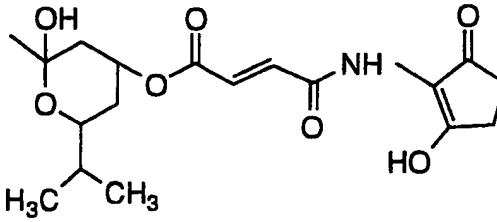
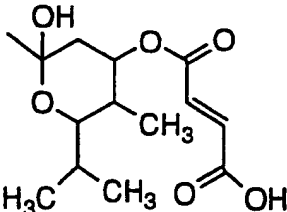
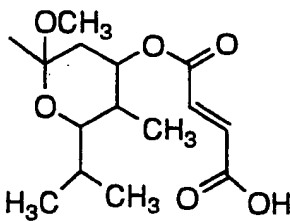
AMENDED CLAIMS

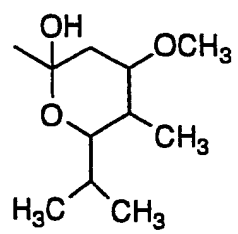
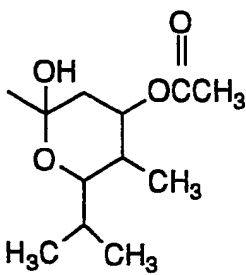
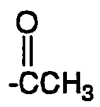
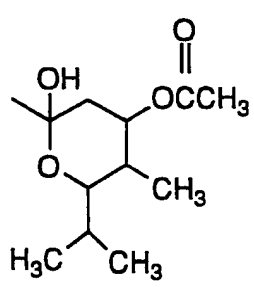
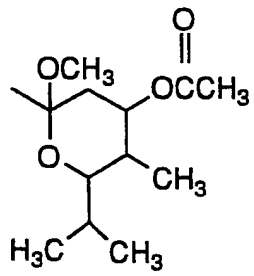
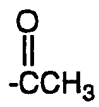
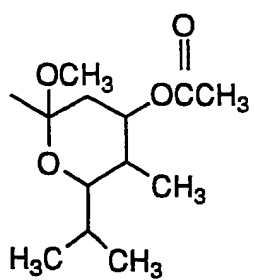
[received by the International Bureau on 20 August 1993 (20.08.93);
original claims 1-7 replaced by amended claims 1-22 (9 pages)]

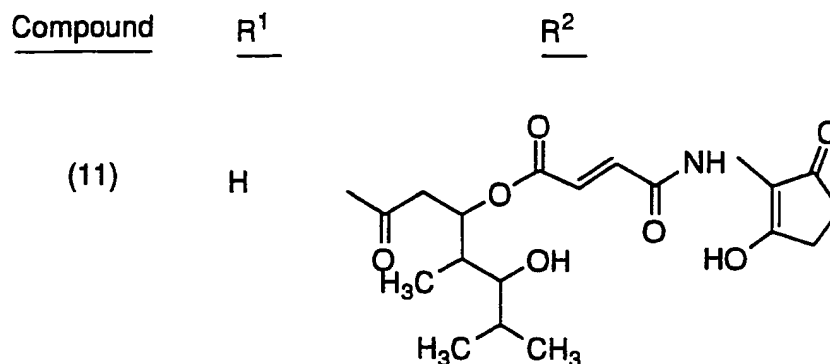
WHAT IS CLAIMED IS:

1. A method of treating tumors of renal cells, colon cells, lung cells or melanoma in a mammal in need thereof, comprising the step of administering to said mammal a pharmacologically effective amount of a compound selected from the group consisting of a bafilomycin of the formula:

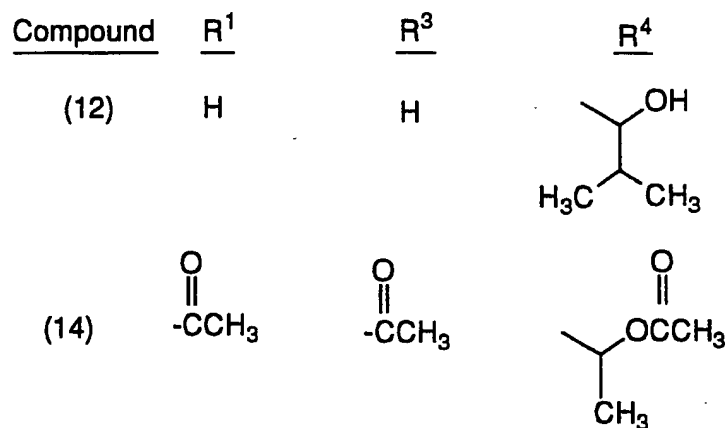
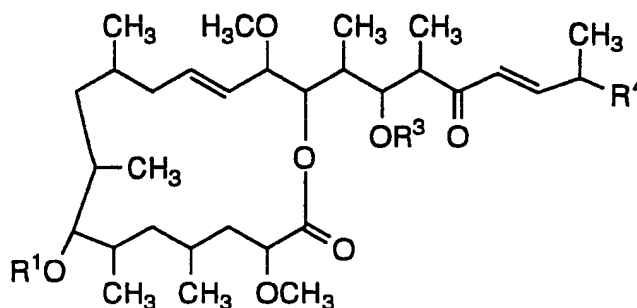


| <u>Compound</u> | <u>R¹</u> | <u>R²</u> |
|-----------------|----------------------|--|
| (1) | H |  |
| (2) | H |  |
| (4) | H |  |
| (5) | H |  |

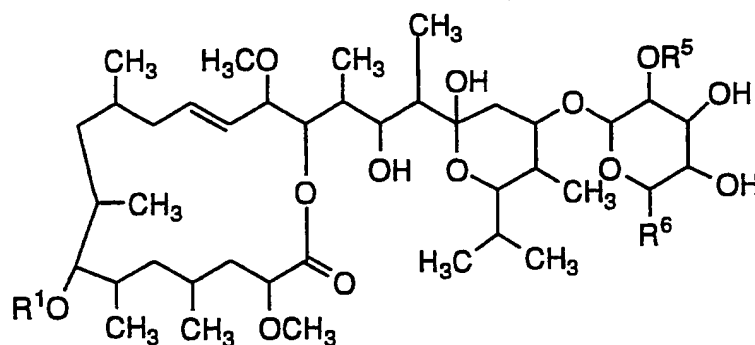
| <u>Compound</u> | <u>R¹</u> | <u>R²</u> |
|-----------------|---|--|
| (6) | H |  |
| (7) | H |  |
| (8) |  |  |
| (9) | H |  |
| (10) |  |  |



2. A method of treating tumors of renal cells, colon cells, lung cells or melanoma in a mammal in need thereof, comprising the step of administering to said mammal a pharmacologically effective amount of a compound selected from the group consisting of a bafilomycin of the formula:

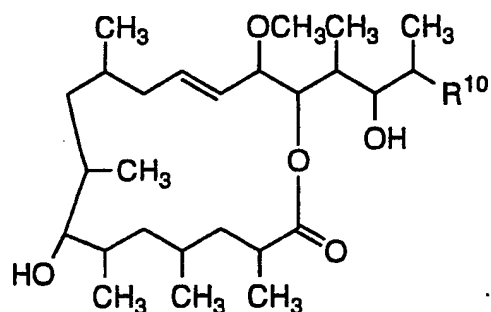


3. A method of treating tumors of renal cells, colon cells, lung cells or melanoma in a mammal in need thereof, comprising the step of administering to said mammal a pharmacologically effective amount of a compound selected from the group consisting of a bafilomycin of the formula:

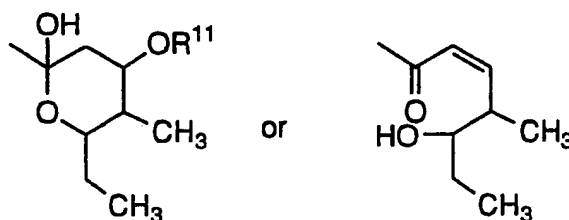


| <u>Compound</u> | <u>R¹</u> | <u>R⁵</u> | <u>R⁶</u> |
|-----------------|---|----------------------|----------------------|
| (15) | H | -CH ₃ | CH ₃ |
| (16) | $\begin{array}{c} \text{O} \\ \parallel \\ \text{CCH}(\text{CH}_3)_2 \end{array}$ | CH ₃ | CH ₃ |
| (17) | H | H | -OH |
| (18) | H | H | -CH ₃ |

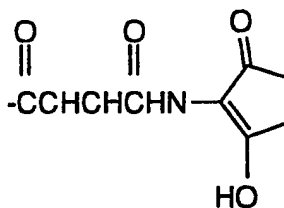
4. A method of treating tumors of renal cells, colon cells, lung cells or melanoma in a mammal in need thereof, comprising the step of administering to said mammal a pharmacologically effective amount of a compound selected from the group consisting of a hygrolidin of the formula



where R¹⁰ is



and R¹¹ is hydrogen, $\text{-C(=O)CH}_2\text{CNH}_2$, $\text{-C(=O)CH}_2\text{COH}$ or



5. The method according to Claim 1 wherein the type of tumor which is treated is a tumor of renal cells in a mammal.

6. The method according to Claim 2 wherein the type of tumor which is treated is a tumor of renal cells in a mammal.

7. The method according to Claim 3 wherein the type of tumor which is treated is a tumor of renal cells in a mammal.

8. The method according to Claim 4 wherein the type of tumor which is treated is a tumor of renal cells in a mammal.

9. The method according to Claim 1 wherein the type of tumor which is treated is a tumor of colon cells in a mammal.

10. The method according to Claim 2 wherein the type of tumor which is treated is a tumor of colon cells in a mammal.

11. The method according to Claim 3 wherein the type of tumor which is treated is a tumor of colon cells in a mammal.

12. The method according to Claim 4 wherein the type of tumor which is treated is a tumor of colon cells in a mammal.

13. The method according to Claim 1 wherein the type of tumor which is treated is a tumor of lung cells in a mammal.

14. The method according to Claim 2 wherein the type of tumor which is treated is a tumor of lung cells in a mammal.

15. The method according to Claim 3 wherein the type of tumor which is treated is a tumor of lung cells in a mammal.

16. The method according to Claim 4 wherein the type of tumor which is treated is a tumor of lung cells in a mammal.

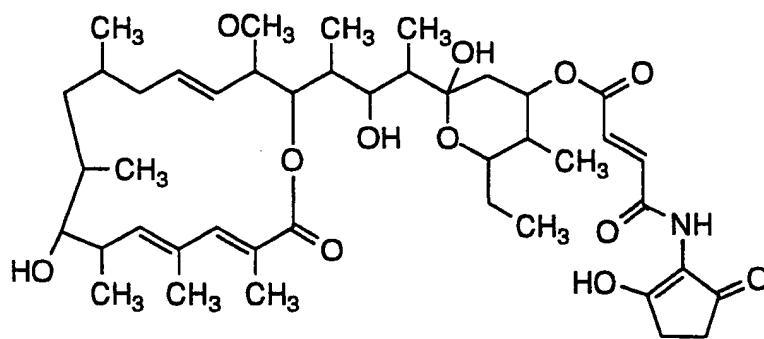
17. The method according to Claim 1 wherein the type of tumor which is treated is a tumor of melanoma in a mammal.

18. The method according to Claim 2 wherein the type of tumor which is treated is a tumor of melanoma in a mammal.

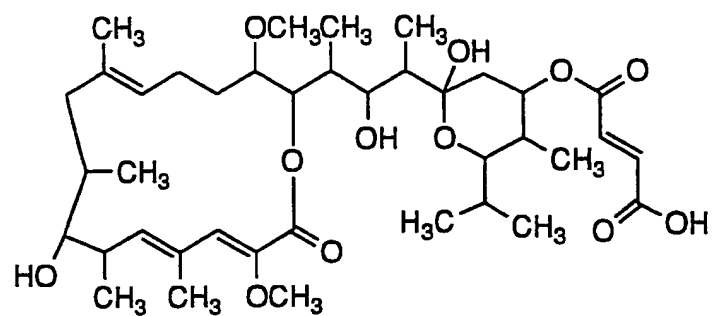
19. The method according to Claim 3 wherein the type of tumor which is treated is a tumor of melanoma in a mammal.

20. The method according to Claim 4 wherein the type of tumor which is treated is a tumor of melanoma in a mammal.

21. The method as claimed in Claim 4, wherein said compound administered to said mammal is



22. The method as claimed in Claim 1, wherein said compound administered to said mammal is



STATEMENT UNDER ARTICLE 19

Applicants have amended Claim 1 as originally filed by rewriting it as four separate independent claims.

New Claims 1-3 claim methods of treatment of tumors utilizing bafilomycins selected from groups of compounds. Support for Claims 1-3 is found on pages 7-12 of the specification as originally filed. Applicants note that the new Claims 1-3 do not include the method of treatment which utilizes the compounds disclosed as antitumor compounds in the J. Antibiotics reference or the JP,A, 1-216927 application cited in the International Search Report.

New Claim 4 is merely part (b) of originally written Claim 1 as an independent claim.

New Claims 5-20 are merely dependent claims similar to originally filed Claims 2-5 which depend on new Claims 1-4 instead of the original Claim 1.

New Claims 21-22 are the same as original Claims 6-7 but now depend on the appropriate new Claims 4 and 1, respectively.

Applicants respectfully contend that no new matter has been introduced by these amendments and that the relevance of the references cited in the international search report may have changed as a result of these amendments.

Applicants, therefore, respectfully request entry of the amendments as specified on the substitute sheets and respectfully assert that the claimed invention as amended discloses an invention that is novel and has an inventive step.

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01N 43/16; A61K 31/35

US CL :514/185, 186, 460

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| X | THE JOURNAL OF ANTIBIOTICS, Vol. XXXVIII, No. 11, "PD 118,576:" "A NEW ANTITUMOR MACROLIDE ANTIBIOTIC" (WILTON), November, 1985, pages 1449-1452. | 1-7 |
| X | JP,A, 1-216927 (AJINOMOTO COMPANY, LTD.) 30 August 1989. See the Abstract and the claims. | 1-7 |

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

| | | |
|---|-----|--|
| * Special categories of cited documents: | *T | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *A* document defining the general state of the art which is not considered to be part of particular relevance | *X* | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *E* earlier document published on or after the international filing date | *Y* | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *Z* | document member of the same patent family |
| *O* document referring to an oral disclosure, use, exhibition or other means | | |
| *P* document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search

11 MAY 1993

Date of mailing of the international search report

24 JUN 1993

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

NATHAN M. NUTTER

Telephone No. (703) 308-2351